Arch Environ Contam Toxicol (2009) 57:405–415
DOI 10.1007/s00244-008-9277-4

Seawater and Shellfish (*Geukensia demissa*) Quality Along the Western Coast of Assateague Island National Seashore, Maryland: An Area Impacted by Feral Horses and Agricultural Runoff

Mary S. Lambert · Gulnihal Ozbay · Gary P. Richards

Received: 22 April 2008 / Accepted: 17 December 2008 / Published online: 9 January 2009

Abstract We evaluated the quality of seawater and ribbed mussels (*Gukensia demissa*) at six sites along the West Coast of Assateague Island National Seashore (ASIS), a barrier island popular with tourists and fishermen. Parameters evaluated were summertime temperature, pH, salinity, dissolved oxygen, total phosphorus, total ammonia nitrogen, and nitrite levels for seawater and total heterotrophic plate counts and total Vibrionaceae levels for the ribbed mussels. Approximately 150 feral horses (*Equus caballus*) are located on ASIS and, combined with agricultural runoff from animals and croplands, local wildlife, and anthropogenic inputs, contribute to nutrient loads affecting water and shellfish quality. The average monthly dissolved oxygen for June was 2.65 mg L^{-1}, below the minimum acceptable threshold of 3.0 mg L^{-1}. Along Chincoteague Bay, total phosphorus generally exceeded the maximum level of 0.037 mg L^{-1}, as set by the Maryland Coastal Bays Program management objective for seagrasses, with a high of 1.92 mg L^{-1} in June, some 50-fold higher than the recommended threshold. Total ammonia nitrogen approached levels harmful to fish, with a maximum recorded value of 0.093 mg L^{-1}. Levels of total heterotrophic bacteria spiked to 9.5×10^6 cells g^{-1} of mussel tissue in August in Sinepuxent Bay, leading to mussels which exceeded acceptable standards for edible bivalves by 19-fold. An average of 76% of the bacterial isolates were in the Vibrionaceae family. Together, these data suggest poor stewardship of our coastal environment and the need for new intervention strategies to reduce chemical and biological contamination of our marine resources.

This study was performed in partial fulfillment of the requirements for a Master's Degree in Natural Resources in the Agriculture and Natural Resources Graduate Program at Delaware State University. The mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

M. S. Lambert · G. Ozbay (✉)
Department of Agriculture and Natural Resources, Delaware State University, 1200 North Dupont Highway, Dover, DE 19901-2277, USA
e-mail: gozbay@desu.edu

G. P. Richards
United States Department of Agriculture, Agricultural Research Service, Delaware State University, Dover, DE 19901, USA

Assateague Island is a barrier island located along the Maryland and Virginia coastline. The Maryland portion was established by Congress as Assateague Island National Seashore (ASIS) on 21 September 1965 for the preservation of natural resources and recreational activities (National Park Service 2001). At that time, a fence was built to separate Maryland and Virginia bands of feral horses (Keiper and Zervanos 1979). The Maryland portion of the island is 35 km long, consisting of 16,187 ha, with the northernmost latitude and longitude of Assateague Island National Seashore's boundary at 38°19′23.65″N, 75°05′39.54″W, and the southernmost boundary located at approximately 38°01′26.45″N, 75°15′04.91″W, and is primarily under the jurisdiction of the National Park Service (NPS); however, 3.2 km of the island is maintained by Assateague State Park (ASP). The southern portion of the island, the Chincoteague National Wildlife Refuge, located in Virginia, is managed by the U.S. Fish and Wildlife Service.

The island's salt marshes are a component of a complex estuarine system and are imperative for the maintenance of regional biodiversity and ecosystem health, while providing habitat for threatened and endangered species (Maryland Department of the Environment 1993). Not only is the island a biologically and ecologically important asset, but also it provides aesthetic and recreational value to those who utilize it. Assateague Island is one of the few remaining undeveloped barrier islands along the Mid-Atlantic coast and is utilized by approximately 2 million visitors annually (National Park Service 2001).

In total, more than 70% of ASIS's area is associated with oceanic and bay waters, which not only facilitates the protection of wildlife, but also provides recreational opportunities. The inshore waters compose a small network of coastal bays, which include the Sinepuxent Bay and Chincoteague Bay systems. These bays extend from the Ocean City Inlet, Maryland, to the Chincoteague Inlet, Virginia, and border the Western coast of Assateague Island. In 1933, a hurricane opened the Ocean City Inlet, which was later stabilized by the U.S. Army Corps of Engineers (Dolan et al. 1977). This event caused the estuary to empty into the Chincoteague Bay and Ocean City Inlet, thereby significantly impacting the salinity and water circulation of the bays (Counts and Bashore 1991). On average, the bays are less than 1.82 m deep and are poorly flushed due to the limited freshwater inflow, reduced flushing through Sinepuxent Bay, and low tidal amplitude (Maryland Department of the Environment 1993). Furthermore, Maryland coastal bays have low dissolved oxygen, excessive nutrients, and elevated levels of fecal coliform bacteria (Maryland Department of the Environment 2005). The volumes of seawater in Sinepuxent Bay, Chincoteague Bay (Maryland portion), and Chincoteague Bay (Virginia portion) are 16.5×10^6, 231.0×10^6, and 143.5×10^6 m^3, respectively (Wazniak et al., 2005c).

The ASIS does not contain agricultural lands; however, areas adjacent to the Sinepuxent Bay and Chincoteague Bay on the mainland of Maryland are prime agricultural areas. Bohlen and Boynton (1998) determined that 36% of the coastal bay's watershed is used for agriculture and estimated that animal production accounts for approximately one-third of the nitrogen and phosphorous entering the coastal bays. Runoff from fertilized farm fields and from wildlife constitutes a nonpoint source of contamination which adds nutrients and bacteria to the water. Feral horses (*Equus caballus*) are an introduced species to ASIS and also affect the salt marsh ecosystem (Hubbard et al. 2004; Reimold et al. 1975). Previous studies (Derlet and Carlson 2002; Hubbard et al. 2004) have shown that horses can increase bacteria and nutrients, which could negatively impact aquatic systems and human health. Therefore, it is possible that horse activity on ASIS may also impact bacteria and nutrients in the water, which is filtered by indigenous ribbed mussels (*Gukensia demissa*). Mussels and other bivalve shellfish serve as sentinels of water quality and some species have been used since 1986 in the National Oceanic and Atmospheric Administration's, National Status and Trends Mussel Watch Program (O'Connor 2002).

Understanding water quality parameters is important because they influence water chemistry and aquatic life. The management objective for the Maryland Coastal Bays Program states that in order to maintain suitable fisheries habitat, an instantaneous dissolved oxygen concentration of 3 mg L^{-1} or a diurnal reading of 5 mg L^{-1} is required. Additionally, phosphorus and nitrogen are important nutrients in aquatic systems; however, at high concentrations they can impair water quality by causing algal blooms, which in turn reduce the amount of dissolved oxygen in the water. Threshold levels of total nitrogen and phosphorous were established by the Maryland Coastal Bays Scientific and Technical Advisory Committee based on living resource indicators, i.e., seagrass. Under these guidelines, total phosphorous levels should remain under 0.037 mg L^{-1} and nitrogen under 0.65 mg L^{-1}, in order to protect seagrass health, while water conditions are considered eutrophic if the TN is 1.0 mg L^{-1} and the TP is 0.1 mg L^{-1} (Wazniak et al. 2005b). Total ammonia nitrogen was previously monitored at ASIS and found to be within the seagrass threshold (< 0.65 mg L^{-1}) on the western portion Sinepuxent Bay and Chincoteague Bay adjacent to ASIS (Wazniak et al. 2005b). In contrast, total phosphorus levels were elevated in Sinepuxent Bay and somewhat less (generally within the seagrass limit) in the Maryland portion of Chincoteague Bay (Wazniak et al. 2005b).

In addition to manure's contribution of organic matter, manure contains many bacteria which could adversely affect water and shellfish quality or safety. The contribution of organic compounds also provides nutrients to support the growth of bacterial populations, while the introduction of nonindigenous bacteria could upset the normal microbiological balance along the coastline. Consequently, the levels of total heterotrophic bacteria and total Vibrionaceae could be altered by feral horse activity and the presence of runoff from farms. Total bacterial counts provide a measure of the relative level of natural and contamination-associated bacteria in shellfish, while high levels imply a likelihood of rapid spoilage of seafoods. Vibrionaceae contain a variety of human and fish pathogens within the genera *Vibrio*, *Photobacterium*, *Plesiomonas*, and *Listonella*. Although members of the genus *Aeromonas* were recently moved from the Vibrionaceae family and placed in a family by itself (the Aeromonidaceae family [Anonymous 2005]), for the purpose of this study, we continue to include *Aeromonas* spp. among the Vibrionaceae. Vibrionaceae are highly divergent bacteria containing free-living and symbiotic species, therefore, not all Vibrionaceae are pathogens.

The primary objectives of this study were (1) to conduct a preliminary summertime survey of water and shellfish quality along the Maryland portion of Assateague Island; (2) to show, to the extent possible, any physical, chemical, or biological effects that horse activity may have on water quality or ribbed mussels inhabiting that area; and (3) to collect baseline information on which to build more substantive research programs to assess the coastal ecosystem along ASIS.

Materials and Methods

Site Description

The western coast of the Maryland portion of Assateague Island constitutes the ASIS and is marked from north to south with kilometer (km) markers ranging from 1 to 35. Six study locations were randomly chosen along the 35 km of ASIS and are referenced henceforth by their km position (5, 10, 14, 17.4, 18.9, and 23.4; Fig. 1). A limited number

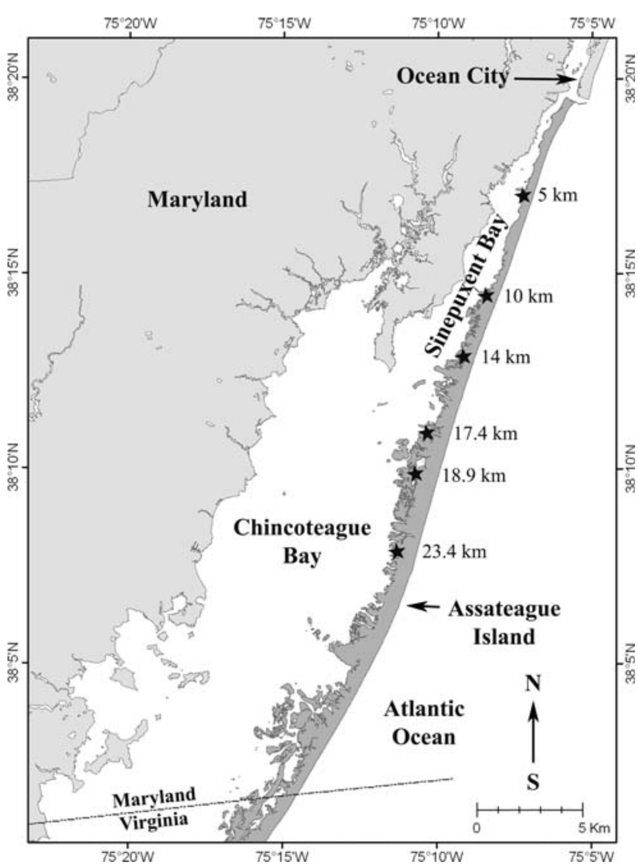

Fig. 1 Six study locations were randomly chosen along the western side of Assateague Island National Seashore for collection of seawater and shellfish (*Gukensia demissa*) samples. Sample sites (indicated by stars) are marked in kilometers (5 to 23.4 km) from the northernmost portion of the island

of ribbed mussels were observed at the north end of the island near Ocean City, Maryland, hence, 60 ribbed mussels were translocated to this site with permission of the ASIS staff and maintained in a wire mesh cage in order to have enough mussels to sample for the study. Sites 17.4, 18.9, and 23.4 were located on Chincoteague Bay and sites 5, 10, and 14 were located on Sinepuxent Bay (Fig. 1). Study sites were marked with wooden stakes inscribed with our research permit number and orange flagging. A four-wheel-drive vehicle was used to access all locations. Site 5 was closed from May to August for the piping plover (*Charadrius melodus*) nesting season (National Park Service 2001) and no samples could be collected at site 23.4 in September, after Hurricane Ernesto, or in October, due to heavy rains and road inaccessibility.

Horse Density

Horse surveys were conducted by ASIS bimonthly (every 2 months) and the data were used to determine horse densities near each site using ArcGIS 9.0 (ESRI, Redland, CA). Assateague Island is divided into zones based on tidal guts (narrow channels formed by receding tides) and drainage areas; therefore, we used the zones where study sites were located to determine horse density.

Water Quality Parameters

General water quality parameters (temperature, salinity, pH, and instantaneous dissolved oxygen [DO]) were monitored twice monthly from the immediate water surrounding the mussels using a YSI Multiprobe (YSI Inc., Yellow Springs, OH). At the same time, 100-mL water samples were collected in clean brown plastic bottles, refrigerated at 4°C, and transported to the laboratory. A HACH DR/2500 Spectrophotometer (HACH, Loveland, CO) was used to measure total phosphorous, total ammonia nitrogen, and nitrite according to the directions provided by the manufacturer (HACH Methods 8190, 8155, and 8507, respectively).

Shellfish Collection

At each study site, 15 to 30 ribbed mussels (*G. demissa*) were collected and stored at ambient temperature in a Styrofoam cooler for same-day analysis. In order to reduce variability, samples were collected shortly after high tide when water had drained from the marsh. Mussels were not stored on ice because cold temperatures cause some Vibrionaceae to enter a viable but nonculturable state (Wright et al. 1996). Ribbed mussels were used as a sentinel species to obtain bacterial counts, rather than water, which is subject to dramatic fluctuations in bacterial levels due to wind, currents, and tides. Although ribbed mussels are not one of the species usually monitored, they, like the more traditional species, also bioconcentrate contaminants from the water column and were at high enough concentrations along Assateague Island to serve as a logical substitute. Samples were collected bimonthly and transported to the Delaware State University Aquatic Sciences Laboratory for analysis.

Total Bacterial Counts and Vibrionaceae Analyses

Mussels were analyzed for both total bacterial counts and total Vibrionaceae counts using the colony overlay procedure for peptidases (COPP assay) (Richards et al. 2005; Richards and Watson 2006). Mussels from each site were washed and shucked and 25 g of meats was pooled and homogenized for 2 min in 225 mL of 0.1% peptone using a Black and Decker ProBlend blender, Series BL1900 (Black and Decker Corp., Towson, MD). Triplicate homogenates were prepared from each site. Once homogenized, serial 10-fold dilutions were made with 0.1% peptone and each dilution was shaken 25 times in a 30-cm arc for 7 s. Then 100 µL of homogenate and dilutions was spread onto plates of tryptic soy agar containing 0.5% added NaCl (TSA-N; 1% total NaCl), and the plates were incubated overnight at 37°C. After incubation, each plate was observed and colonies on one countable plate, generally containing 30 to 100 colonies from each dilution series, were enumerated and the total bacterial counts were recorded. Total bacterial levels per gram were calculated.

The same plate used for total bacterial counts was overlaid for 10 min at 37°C with a cellulose acetate membrane (Sartorius AG, Goettingen, Germany) previously soaked in 20 mM Tris buffer, pH 9.5, containing 250 µM of the synthetic fluorogenic substrate L-lysyl-7-amino-4-trifluoromethylcoumarin (MP Biomedicals, Solon, OH). Enzymes present in colonies of Vibrionaceae family members cleave the substrate, which causes fluorescent foci on the membranes upon exposure to 364-nm (long-wavelength) ultraviolet light (Richards et al. 2005). Fluorescent foci were enumerated and the total Vibrionaceae per gram of shellfish tissue was calculated.

Statistical Analyses

The data were analyzed using regression analyses, repeated-measure ANOVA, and Kruskal-Wallis ANOVA of ranks (Systat Software Inc., San Jose, CA). When statistical differences were found, pairwise comparisons were conducted using a post hoc Tukey test. For all statistical analyses, a confidence interval of 95% and α level of 0.05 were used.

Results

Horse Density

The mean horse densities were 0.29 and 0.56 horse ha^{-1} in July and September, respectively (Table 1). Study site 10 had the highest average density of horses (0.65 ha^{-1}), while the lowest average horse density during the study was at site 23.4, which had 0.23 horse ha^{-1}. The highest horse density during the survey was for site 17.4 in September (0.99 horse ha^{-1}).

Water Parameters

The average monthly water temperatures for all sites ranged from 19.7°C in October to 29.9°C in August (Fig. 2a). Overall, water temperature increased from June to August and started decreasing in September, with the lowest water temperatures occurring in October. Water temperature changed significantly ($p < 0.05$, df = 25) from June to October. The water temperature in August (30°C) was significantly warmer than the water temperature in September (26°C) and October (20°C; $Q = 4.20$ and 3.33, respectively). The October water temperature was significantly cooler than the mean water temperature in July (28°C; $Q = 2.89$). There were no significant differences in temperature among the six study sites during any given month.

The average DO at each study site varied over the duration of the study (Fig. 2b). The average monthly DO for all the study sites ranged from 2.7 mg L^{-1} in June to 8.7 mg L^{-1} in October. The mean DO generally increased from June (2.7 mg L^{-1}) to July (4.5 mg L^{-1}), and to August (5.6 mg L^{-1}) and was highest in September (8.3 mg L^{-1}) and October (8.8 mg L^{-1}); however, site 5 data were incomplete because of inaccessibility of the site due to the piping plover (*Charadrius melodus*) nesting season, which runs from May to August. Site 23.4 was also

Table 1 Density of feral horses (*Equus caballus*) along Assateague Island National Seashore at each study site for July and September 2006

Site	Density (horse ha^{-1})		
	July	September	Mean per site
5	0.39	0.47	0.43
10	0.70	0.59	0.65
14	0.20	0.62	0.41
17.4	0.00	0.99	0.50
18.9	0.00	0.73	0.37
23.4	0.45	0.00	0.23
Monthly mean	0.29	0.56	

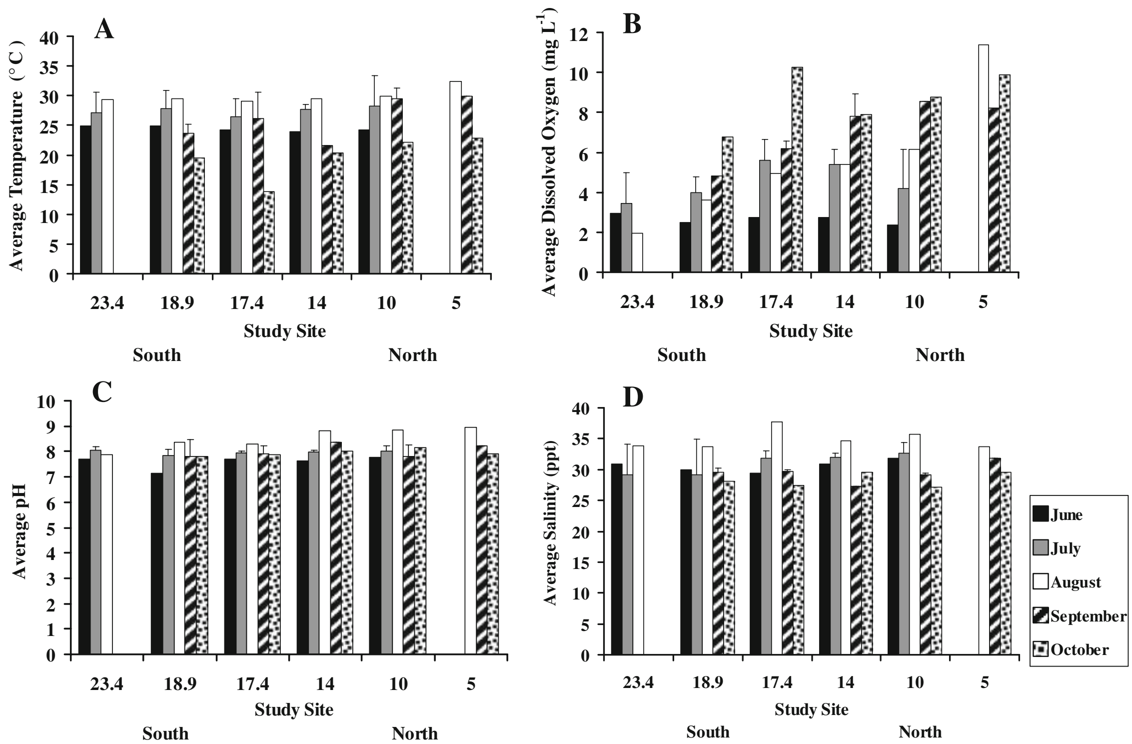

Fig. 2 Average seawater conditions along Assateague Island National Seashore, Maryland, from June to October 2006. **a** Temperature, **b** dissolved oxygen, **c** pH, and **d** salinity. Bars represent the mean of triplicate assays ± standard deviation. The key to the right of panel **d** is for all panels (**a–d**)

inaccessible because of heavy rains in September and October (Fig. 2b). October had significantly higher DO levels compared with the mean June and July data ($p = 0.001$ and 0.015, respectively) and September had significantly higher DO levels than June ($p = 0.009$) (Fig. 2b). The DO increased from sites 23.4 to 5, south to north, respectively. Overall, there was an inverse relationship between water temperature and dissolved oxygen.

During most months, sites met the Maryland Coastal Bays Program management objectives for DO, however, sites 10, 14, 17.4, 18.9, and 23.4 were deficient in June (instantaneous DO readings ranged from 2.36 to 2.96 mg L^{-1}) and site 23.4 was deficient in August (DO = 1.97 mg L^{-1}). The management objective states that in order to maintain a suitable fisheries habitat, an instantaneous DO concentration of 3 mg L^{-1} or a diurnal reading of 5 mg L^{-1} is required. The low DO levels in June could have been caused by sampling early in the morning, as stated by Wazniak et al. (2005a), who showed that the lowest DO levels occurred in the early to mid-morning hours. The data collected over the duration of this study show lower DO levels than those reported during the Maryland Coastal Bays Assessment. According to the 2004 Ecosystem Health Assessment, there was only one site in the Sinepuxent Bay that did not meet the DO threshold requirement. This site was located at a commercial harbor near the Ocean City Inlet. Furthermore, most of Chincoteague Bay, except for some coves, met the DO requirements (Maryland Department of the Environment 2005).

Along the island, the overall pH ranged from 7.13 (site 18.9) to 8.92 (site 5) (Fig. 2c). The highest average monthly pH occurred in August (8.52), and the lowest average monthly pH level occurred in June (7.58). In general, average pH increased steadily from June (7.59) to July (7.97) to August (8.52) and a decreasing trend was observed from September (8.02) to October (7.95). The decline in pH may be from rain events in September and October (20.21 and 16.07 cm, respectively). The pH was significantly higher in August than in June, July, and October ($p = 0.001$, 0.018, and 0.018, respectively), but there were no significant differences between study sites ($p = 0.258$).

Salinity levels ranged from 27.2 to 37.4 ppt at sites 10 and 17.4, respectively (Fig. 2d). Three readings were obtained each time mussel samples were collected. The values in Fig. 2d are the averages of those values. The highest average monthly salinity occurred in August (34.9 ppt) and the lowest average salinity occurred in October (28.3 ppt). August also had the highest water temperatures and the highest pH (Fig. 2). A post hoc Tukey test found significant differences in mean salinities between August and October, September, June, and July (all p's < 0.001, df $= 25$), as well as between July and October ($p = 0.048$). The differences in salinity between study locations were not great enough to be statistically significant ($p = 0.992$). Overall, the trends in salinity were homogeneous across the different sites because of the lack of freshwater inflow (Maryland Department of the Environment 2005). Increased salinity during August can be accounted for by the increased water temperature, which increases evaporation, and the small amount of precipitation (2.64 cm). Most readings were the same during each sampling period at each site except in July, where the standard deviation was the highest.

Waters were also analyzed for total phosphorus (TP), total ammonia nitrogen (TAN), and nitrite. Most TP readings exceeded the maximum levels of 0.037 mg L^{-1}, as described in the seagrass management objective set by the Maryland Coastal Bays Program (Fig. 3a). The highest TP concentrations were observed in June for the three southern sites: 1.92, 1.25, and 1.84 mg L^{-1} for sites 23.4, 18.9, and 10, respectively (Fig. 3a). These levels were up to 50-fold higher than the maximum levels proposed for healthy seagrass and indicate highly eutrophic conditions (Wazniak et al. 2005b). Conditions are considered eutrophic when TP levels reach 0.1 mg L^{-1} or higher. Phosphorus levels decreased in September from the south to the north of the island (0.28 to 0.08 mg L^{-1}) for site 18.9 to 10, respectively. Furthermore, in July TP increased from site 23.4 (0.3 mg L^{-1}) to site 14 (0.9 mg L^{-1}). In the 2004 Ecosystem Health Assessment Report, Chincoteague Bay had

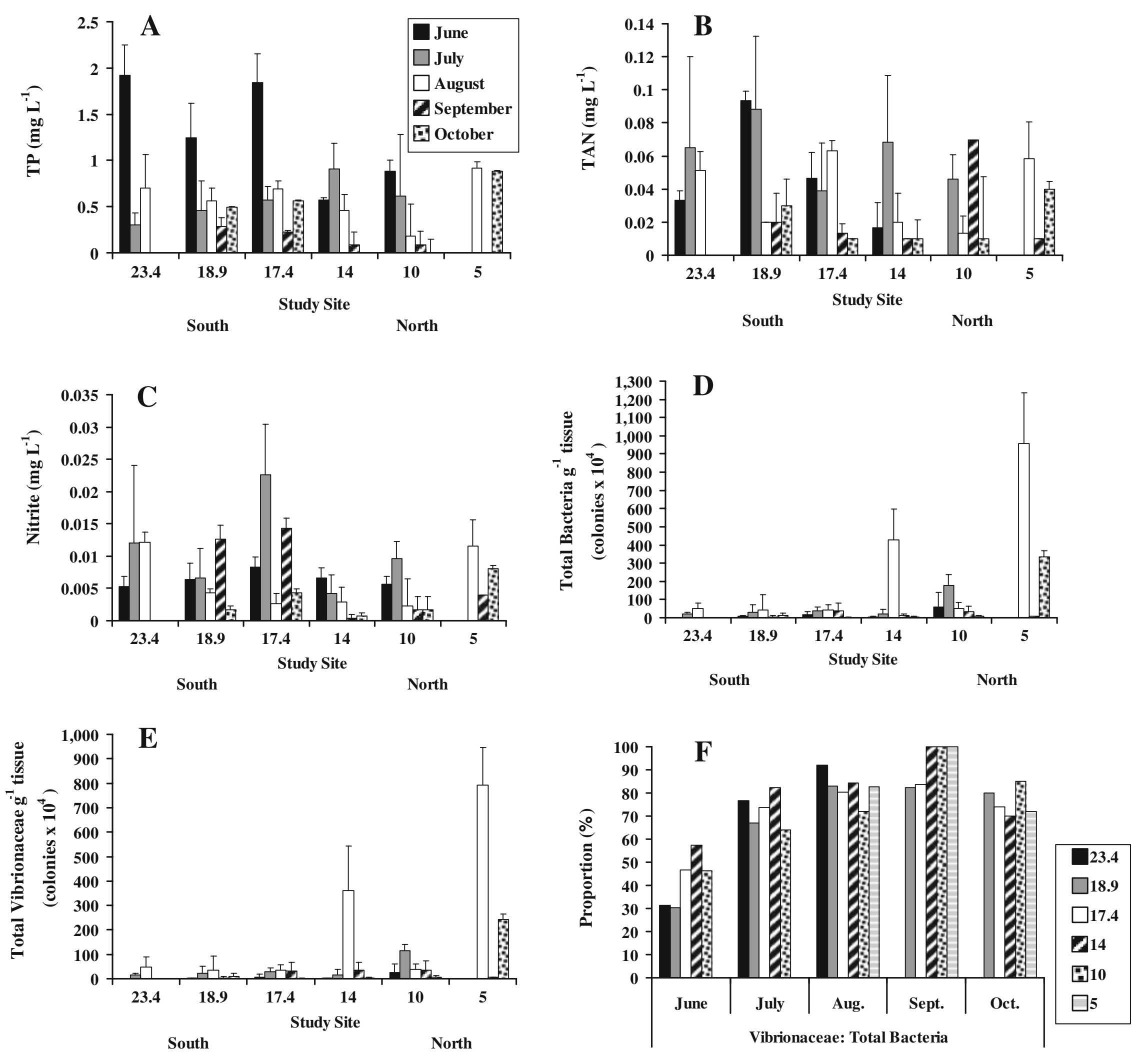

Fig. 3 Nutrient and bacterial levels along Assateague Island National Seashore from June to October 2006. **a** Average total phosphorus, **b** total ammonia nitrogen, **c** nitrite, **d** total heterotrophic bacteria, **e** total Vibrionaceae, and **f** proportion of total Vibrionaceae to total heterotrophic bacteria in seawater (**a–c**) and ribbed mussels (*Gukensia demissa*; **d–f**). Bars in **a–e** represent the mean of triplicate assays ± standard deviation. The key shown in panel **a** is applicable to panels **a–e**; the key in panel F indicates specific study sites for panel **f**

the highest TP concentrations (1.1 to 1.7 mg L^{-1}) (Wazniak et al. 2005b), which were similar to the concentrations found in this study. The higher TP concentration might be caused by runoff from the mainland, since the northern part of Sinepuxent Bay is mainly developed, but the area closer to the access bridge to Assateague Island, between site 10 and site 14, is primarily agriculture and forest. Also, south of the bridge, near site 14, there is a mixture of forest, development, and agriculture. Phosphorous enrichment appears to be a problem throughout the bays but is especially high along Chincoteague Bay.

Total ammonia nitrogen (TAN) concentrations fluctuated over the duration of the study ($p = 0.037$, df = 25) (Fig. 3b). The lowest concentration was 0.01 mg L^{-1}, which occurred in September at site 14 and in October at sites 10, 14, and 17.4, whereas the highest concentration occurred in June at site 18.9 (0.093 mg L^{-1}). No obvious trends of increasing TAN along the island or between sites were observed ($p = 0.652$, df = 20). Our analytical technique monitored both ionized (NH_4^+) and nonionized (NH_3) ammonia. We call it "total ammonia nitrogen." NH_3 is the principal form of toxic ammonia and its toxic levels are both pH and temperature dependent. Toxicity increases as pH decreases and as temperature decreases. Total ammonia nitrogen, which can be toxic to fish species, was within normal levels at most study sites. Toxicity levels for nonionized ammonia depend on the individual species; however, levels below 0.05 mg/L are considered safe.

Total nitrogen (TN) levels were not collected in this study, but data obtained from the Maryland Coastal Bays Health Assessment shows that overall TN levels were below the seagrass threshold requirement (< 0.64 mg L^{-1}) in Sinepuxent Bay, and most stations along Assateague Island in Chincoteague Bay met the TN seagrass threshold (Wazniak et al. 2005b).

Nitrite levels ranged from 0.0003 mg L^{-1} in September at site 14 to 0.023 mg L^{-1} in July at site 17.4 (Fig. 3c). June levels had the least amount of variation between sites (0.005 to 0.008 mg L^{-1}). No limits on nitrite levels have been set for marine waters, but average nitrite levels were below the maximum level (1 mg L^{-1}) set by the U. S. Environmental Protection Agency (1989) for regulating public drinking water supplies.

Shellfish Microbiology

Total Heterotrophic Bacterial Counts

Total bacterial counts ranged from a low of 1.7×10^4 colony forming units (cfu) g^{-1} mussel tissue at site 23.4 in June to a high of 9.6×10^6 cfu g^{-1} mussel tissue at site 5 in August (Fig. 3d). In contrast, safe levels of total heterotrophic bacteria for oysters, clams, and edible mussels about to enter the marketplace have been set by the U.S. Food and Drug Administration at $\leq 5.0 \times 10^5$ cfu g^{-1} of shellfish meat, thus the level at site 5 in August is 19-fold higher than the acceptable standard for consumable shellfish. The three southern sites showed no significant differences ($p > 0.05$) in the mean total bacterial counts among the study locations. Total bacterial levels increased from June to August and decreased in September and October at each study site except site 5, which had higher levels of bacteria in October than in September (3.3×10^6 vs 7.1×10^3 cfu g^{-1} mussel tissue, respectively) (Fig. 3d). Mean bacterial levels were lower at the sites located in Chincoteague Bay (sites 17.4, 18.9, and 23.4) and higher in Sinepuxent Bay (sites 5, 10, and 14). In addition, bacterial loads were significantly higher in August than in any other month, which is probably because of increased temperature and nutrients in the water during the peak of summer. Wright et al. (1996) evaluated oysters from Chesapeake Bay and found total heterotrophic bacterial levels of 8.5×10^2 to 4.6×10^5 cells g^{-1} oyster tissue. In this study, the total heterotrophic bacterial counts spiked in August, to 9.6×10^6 cells and 4.3×10^6 cells g^{-1} mussel tissue at sites 5 and 14, respectively. Not only were there differences in bacterial levels between months, but in the northernmost site, bacterial loads were significantly higher than at any other site in August and October.

Land cover data were used to determine land use of the Sinepuxent Bay and Chincoteague Bay watersheds (Maryland Department of the Environment 2005). The Sinepuxent Bay watershed has a total of 5548 ha and is composed of 12% urban, 6.2% agriculture, 17% forest, and 58.1% water/wetland, with the remaining area (6.6%) identified as other (Maryland Department of the Environment 2005). The Chincoteague Bay watershed is 36,133 ha, which is composed of 19.2% forests, 15.9% agriculture, 63.1% water/wetlands, 0.8% urban, and 1% other (Maryland Department of the Environment 2005). At site 14 there was some adjacent development, such as a golf course, which may influence nutrient levels. Site 5 could experience spikes in bacterial levels due to its proximity to population centers and beach activities around Ocean City, Maryland.

Vibrionaceae

Total Vibrionaceae counts followed trends similar to those for total bacterial counts along the island and throughout the duration of the study. Counts were the highest at sites 14 and 5 in August, 3.6×10^6 and 7.9×10^6 cfu g^{-1} mussel tissue, respectively (Fig. 3e). The lowest Vibrionaceae counts occurred in June and at sites 17.4, 18.9, and 23.4, which had varying levels of horse activity, less human activity than sites 10 and 14, and less mainland development. Low counts for June ranged from 5.3×10^3 to 7×10^4 cfu g^{-1} mussel tissue. For October, the highest count occurred at the northernmost site. Bacterial levels decreased as the water temperature decreased.

Vibrionaceae counts from the sites located in Chincoteague Bay did not exceed 5×10^5 cfu g^{-1} mussel tissue. Furthermore, Vibrionaceae counts were below 1×10^6 cfu g^{-1} mussel tissue for all sites and months, except for spikes in counts at sites 5 and 14 in August, site 5 in October, and site 10 in July. In contrast, total Vibrionaceae counts from Delaware Bay have been shown to exceed 1×10^6 cfu g^{-1} shellfish during the summer months and to exhibit similar spikes (Gary Richards; unpublished data).

The proportion of Vibrionaceae to total bacteria ranged from 30% to 100%, with an overall average of 76% (Fig. 3f). The lowest proportion of Vibrionaceae to total bacteria occurred in June (42% Vibrionaceae) and increased until September when all of the isolates were Vibrionaceae at the three northernmost sites (Fig. 3f). In October, the proportion of Vibrionaceae began to decrease. In October, Vibrionaceae constituted approximately 76% of the total bacteria identified and enumerated in each sample. Only recently, with the development of the COPP assay (Richards et al. 2005), has the ability to quantify total Vibrionaceae levels become possible. Pathogenic and nonpathogenic Vibrionaceae are known to increase dramatically in water and shellfish during the summer months and then to decrease to almost-nondetectable levels during the winter. The spikes in Vibrionaceae counts during August suggest a period when local water conditions or shellfish were favorable to outgrowth or infection by these bacteria.

There were clear pin-point colonies that were difficult to detect on the TSA-N plates used for the enumeration of total bacteria; however, once the colonies were overlain and the membranes placed under ultraviolet light, pin-point colonies produced fluorescent foci which were easily enumerated. Some of these colonies appeared to be *Shewanella*, a genus of pathogenic and nonpathogenic bacteria once proposed to be placed in the Vibrionaceae family (MacDonell and Colwell 1985). *Shewanella* species from Delaware Bay oysters and seawater were recently shown to produce fluorescence in the COPP assay (Richards et al. 2008).

Discussion

In 1996, Sinepuxent Bay and Chincoteague Bay were listed under Section 303(d) of the Clean Water Act as impaired by virtue of their DO, nutrient, and fecal coliform levels. In 2002, the Maryland Department of the Environment (2005) removed the dissolved oxygen impairments under the guidance of the EPA because DO was not considered an impairing substance. Water temperature increased over the late spring and early summer months, June to August, and declined in September and October. These temperatures reflect the normal seasonal warming during summer and slight cooling during early autumn. The low DO readings, especially noticeable in June for the three southernmost sites, corresponded with high TP levels.

Although ASIS does not contain agricultural lands, areas adjacent to the Sinepuxent Bay and Chincoteague Bay on the mainland of Maryland are prime agricultural areas. The highest levels of phosphorous were found at the three southern sites along Chincoteague Bay. Bohlen and Boynton (1998) determined that 36% of the coastal bay's watershed is used for agriculture and estimated that animal production accounts for approximately one-third of the nitrogen and phosphorous entering the coastal bays. Interestingly, the highest phosphorous levels obtained during this study were in June, which is shortly after the time in which farmers fertilize their fields. The high phosphorous levels in June are not likely from feral horses, since horses are present throughout the year and should contribute to relatively constant levels of nutrients, subject to some fluctuations from rain events.

Water temperature and salinity are not expected to seriously alter the levels of phosphorous or nitrogenous compounds in the water; however bacteriological levels would be expected to increase dramatically with increasing water temperatures and, to a lesser degree, with changing salinities. Dramatic increases in total bacterial counts and Vibrionaceae typically occur during the warm summer months in tributaries around the world. Such increases occur in areas regardless of the presence or absence of horse populations. Nevertheless, the addition of nutrients and manure-related bacteria to the bays would be expected to influence to some extent the peak levels of indigenous, wildlife-associated, and anthropogenic bacteria on ASIS, since nutrient-rich waters may promote the propagation of natural and contributed bacterial populations. Since agricultural runoff, rather than horses, may be the principal contributor to the high nutrient levels in the bays, additional, longer-term studies are needed to determine the influence of agricultural runoff on water and shellfish quality and safety along ASIS.

The carrying capacity of horses on Assateague Island was established by Keiper and Zervanos (1979) as 244 animals, based on the utilization of primary forage plants, and they recommended that a population of 120–150 horses be maintained. Today, approximately 150 horses inhabit ASIS. Horses use six different habitats along the island: sandy beaches, to escape biting insects; dunes, which provide forage; inner dunes, which provide grassy areas for grazing; the shrub zone, primarily for traveling between areas; areas with trees, which are resting areas at night and contain permanent freshwater holes; and the salt marsh (Keiper 1978). Large bands of horses spend more time in salt marshes than smaller bands (Keiper and Zervanos 1979). Redman and Goodwin (1999) found that horse manure was randomly distributed, except where stud piles (large piles of manure produced by stallions to mark their territory) existed. Furthermore, most (84%) of the horses were observed defecating while grazing during the summer months (Redman and Goodwin 1999). Based on previous research and personal observations, mares and juvenile feral horses on Assateague Island show random eliminative behavior, whereas males show nonrandom defecation when marking their territory. Horses defecate every 1.25 to 1.5 h when grazing and approximately every 3 h when resting. Redman and Goodwin (1999) and Lamoot et al. (2004) found that horse manure is randomly distributed, unless they are confined at high densities. Based on a vegetation map developed for ASIS during the 1990 s, approximately 70% of the salt marsh was classified as low marsh and was submerged during high tide, whereas 30% was high-salt marsh that was not affected by tides (personal communication, Cark Zimmerman, ASIS). Horses were found to spend an average of 46.9% of their time in salt marsh habitats during the summer months (Zervanos 1978) and an average of 39.8% during the winter months (Zevanos and Keiper 1979). In that research, the authors defined salt marsh very broadly, including both high and low marsh types.

Horses weighing 454.6 kg can produce 8164.6 kg of manure $year^{-1}$, which is approximately 22.7 kg day^{-1} (Davis and Swinker 1996). The ratio of nitrogen to phosphorus in horse manure ranges from 2.9:1 to 6.7:1 and the average N:P ratio is 4.7:1 (Mackenthun 1999). On average, horses produce 8.62 kg of nitrogen and 6.35 kg of phosphorus ton^{-1} of manure (Davis and Swinker 1996). According to Keiper (1978), horses urinate every 2 to 2.5 h and stallions urinate on the spots where their mares or stallions from other bands have urinated. Additionally, Hubbard et al. (2004) reported that on average horses produce 10 kg of urine daily. As high tide recedes, water containing manure, urine, and nutrients drains off the marshes into the bays. We expected that areas with higher horse densities should have more manure and urine production, which would likely be the case if the horses were retained within those areas. However, the high mobility of horses could mean that animals which grazed and defecated in an area one day may have moved by the time the horse density data were collected. Therefore, it was not scientifically valid to directly correlate horse density data with water or shellfish quality parameters. Areas without active horse activity would still have a persistent contamination of water and shellfish by manure, as a consequence of tidal and rain-associated runoff. We also found that all sampling sites were impacted by horses, thus there were no pristine areas within the watershed to serve as horse-free control sites from which baseline data could be obtained.

Horse manure was widely scattered throughout the island, some underwater, some in intertidal areas which would be picked up and washed away by the seawater during high tides, some at higher elevations not directly impacted by tides but along tidal streams, and some at higher elevations that were not impacted by tides. These areas of higher elevation would contribute to contamination of the bays primarily through storm runoff. The varying topography on the island (some marsh/pasture areas impacted by tidal influx, some areas populated with trees, and other open areas at varying elevations) made it impossible to discern what amount of manure would enter the bays or when it would enter (after light rains, heavy rains, etc.). It became apparent that we could not accurately determine what amount of the manure actually entered the bays and that correlations of horse or manure density and water quality were not possible. Consequently, we conclude that water quality parameters above recommended limits are likely influenced by the horse population, but may also be impacted by other wildlife, as well as from contributions from anthropogenic sources and agricultural runoff.

Both phosphorus and nitrogen are important nutrients in aquatic systems; however, at high concentrations they can impair water quality by causing algal blooms, which in turn reduce the amount of DO in the water. Horse activities and agricultural practices on the mainland likely contributed to the low DO and the high TP and TAN levels. If conditions worsen, it is anticipated that more sites along the island will be closed for recreational activities, while toxic effects of the chemicals may further affect sea life in the area. TAN was monitored since it is highly toxic to fish and nitrite levels were determined since nitrite is an intermediary between ammonia and nitrate.

Increased nutrients from runoff might increase naturally occurring aquatic bacteria such as Vibrionaceae, which are one of the most predominant families of marine bacteria (Simidu and Tsukamoto 1985; Croci et al. 2001). Free-living and symbiotic species within this family are associated with aquatic organisms ranging from plankton to fish (Simidu and Tsukamoto 1985). Vibrionaceae are important to understand and monitor because the genera *Vibrio*, *Aeromonas*, *Photobacterium*, and *Plesiomonas* contain human and wildlife pathogens. Since molluscan shellfish are bioconcentrators, capable of filtering bacteria from the water and concentrating them within their tissues, bivalve mollusks serve as hosts of bacterial pathogens within the Vibrionaceae family, thus shellfish consumption, either raw or lightly cooked, can lead to illness and death. The occurrence of some pathogenic Vibrionaceae, like *V. vulnificus*, can be enhanced by lower salinity (7 to 16 ppt) and higher temperatures (> 20°C) (Wright et al. 1996).

Impairment of the water quality bordering ASIS suggests an influence of feral horse activity, but the highly migratory nature of the horses, the widespread distribution of manure, and intermittent rain events make it difficult to directly associate the presence of horses with any specific contamination event. Using the Davis and Swinker (1996) data for domestic horses, the average amount of manure produced daily by a feral horse was estimated. Horses on Assateague Island weigh approximately 362.8 to 408.2 kg, therefore, it is estimated that an individual horse produces between 17.9 to 20.1 kg of manure day^{-1}. For a herd of 150 horses spread throughout ASIS, this equates to approximately 2850 kg of manure daily. Hubbard et al. (2004) determined that a ton (909 kg) of manure contains 8.82 kg of nitrogen and 6.35 kg of phosphate, thus the horses of Assateague Island would deposit ~27.6 kg of nitrogen and 19.9 kg of phosphorus each day. When we consider that only about one-half of the nutrient runoff goes into the Chincoteague and Sinepuxent Bays, while the other half drains into the Atlantic Ocean from the eastern side of the island, this contribution to the bays seems small compared to the amount of nitrogen and phosphorous that likely enters the watershed from agricultural sources, particularly during peak fertilization and runoff periods.

In summary, seawater from some sites bordering the western coast of ASIS suffers from low DO and excessive TP levels, as measured against thresholds set to maintain healthy seagrass beds. Both low DO and high TP indicate eutrophic conditions. Levels of total heterotrophic bacteria spiked in ribbed mussels in August at two sites in Sinepuxent Bay, leading to mussels which exceeded acceptable standards for edible bivalves by up to 19-fold. The vast majority (up to 100%) of the bacterial isolates were in the Vibrionaceae family. Sources of water contamination include agricultural runoff from animals and crops along the Maryland and, to a lesser extent, the Virginia mainland, feral horses inhabiting ASIS, local wildlife, and anthropogenic inputs. The impact of feral horses on seawater and shellfish quality at the various sites could not be directly assessed due to the migratory nature of the horses, the inability to quantify manure loads, particularly since many of the grazing areas are underwater during high tide, and the uncertainty of how much of the manure at higher elevations enters the water during rain events. Agricultural runoff from recently fertilized farmlands on the mainland likely contributed to major spikes in TP levels in Chincoteague Bay in June. Together, these data suggest poor stewardship of our coastal environment and the need for new intervention strategies to reduce chemical and biological contamination of our marine resources.

Acknowledgments This study was supported by funds under a USDA Cooperative State Research, Education, and Extension Service Grant 2004-38820-15154 and a USDA-Evans Allen Grant, and by intramural funds from the USDA Agricultural Research Service. We wish to thank Drs. Dennis McIntosh and Kevina Vulinec for guidance and suggestions, and Michael Watson and Kesha Braunskill for their technical assistance. We would like to gratefully acknowledge the staff at the Assateague Island National Park, particularly Carl Zimmerman and Brian Sturgis, who helped with the logistics of this study and provided assistance during the field season, and Allison Turner, who conducted the horse distribution surveys.

References

Anonymous (2005) Minutes. International committee on systematics of prokaryotes subcommittee on the taxonomy of vibrionaceae. Int J Syst Evol Microbiol 55:539–542. doi:10.1099/ijs.0.63519-0

Bohlen C, Boynton W (1998) Status and trends of eutrophication, chemical contamination, habitat loss/modification, pathogens and living resources in Maryland Coastal Bays. MCBP 97–01. Maryland Coastal Bays Program. Maryland Department of Natural Resources, Berlin

Counts CL, Bashore TL (1991) Molluscs of Assateague Island, Maryland and Virginia: a reexamination after seventy five years. Veliger 34:214–221

Croci L, Serratore P, Cozzi L, Stacchini A, Milandri S, Suffredini E (2001) Detection of Vibrionaceae in mussels and in their seawater growing area. Lett Appl Microbiol 32:57–61. doi:10.1046/j.1472-765x.2001.00855.x

Davis JG, Swinker AM (1996) Horse manure management. Colorado State University Cooperative Extension. Livest Ser 1:219

Derlet RW, Carlson JR (2002) An analysis of human pathogens found in horse/mule manure along the John Muir Train in Kings Canyon and Sequoia and Yosemite National Parks. Wilderness Environ Med 13:113–118

Dolan RG, Hayden B, Heywood J (1977) Atlas of environmental dynamics, Assateague Island National Seashore, Washington, DC. Natural Resource Report 11. U.S. Department of Interior, National Park Service, p 39

Hubbard RK, Newton GL, Hill GM (2004) Water quality and the grazing animal. J Anim Sci 82:255–263

Keiper RR (1978) The behavior, ecology, and social organization of the feral ponies of Assateague Island. National Park Service Transaction and Proceedings Series 5, pp 369–371

Keiper RR, Zervanos SM (1979) Seasonal home ranges and activity patterns of feral Assateague Island ponies. In: Symposium on the ecology and behavior of wild and feral equids, University of Wyoming, pp 3–14

Lamoot I, Callebaut J, Degezelle T, Demeulenaere E, Laquiere J, Vandenberghe C, Hoffman M (2004) Eliminative behavior of free-ranging horses: Do they show latrine behavior or do they defecate where they graze? Appl Anim Behav Sci 86:105–121. doi:10.1016/j.applanim.2003.12.008

MacDonell MT, Colwell RR (1985) Phylogeny of the family Vibrionaceae and recommendations for two new genera: *Listonella* and *Shewanella*. Syst Appl Microbiol 6:171–182

Mackenthun KM (1999) Phosphorus concentration in manure. Water Environ Technol 11:6

Maryland Department of the Environment (1993) Maryland's coastal bays: an assessment of aquatic ecosystems, pollutant loadings, and management options. Chesapeake Bay and Special Operations Branch, Baltimore

Maryland Department of the Environment (2005) Water quality analyses of fecal coliform for eight basins in Maryland:

Assawoman Bay, Sinepuxent Bay, Newport Bay, and Chincoteague Bay in Worcester County; Monie Bay in Somerset County; Kent Island Bay in Queen Anne's County; Rock Creek in Anne Arundel County; and Langford Creek in Kent County. Maryland Department of the Environment, Baltimore, pp 5–8, 13–17

National Park Service (2001) Management and monitoring of the piping plover (*Charadrius melodus*) at Assateague Island National Seashore, Maryland. Maryland Department of Natural Resources, Wildlife, and Heritage Service, Annapolis

O'Connor TP (2002) National distribution of chemical concentrations in mussels and oysters in the USA. Mar Environ Res 53:117–143. doi:10.1016/S0141-1136(01)00116-7

Redman P, Goodwin D (1999) Grazing and defecation behavior of a bachelor group of Przewlaski horses (*Equus przewlaskii*) under free-ranging and enclosed conditions. Equine Vet J Suppl 28:68–69

Reimold RJ, Linthurst RA, Wolf PL (1975) Effects of grazing on a salt marsh. Biol Conserv 8:105–125. doi:10.1016/0006-3207(75)90036-1

Richards GP, Watson MA (2006) A simple fluorogenic method to detect *Vibrio cholerae* and *Aeromonas hydrophila* in well water for areas impacted by catastrophic disasters. Am J Trop Med Hyg 75:516–523

Richards GP, Watson MA, Parveen S (2005) Development of a simple and rapid fluorogenic procedure for identification of Vibrionaceae family members. Appl Environ Microbiol 71:3524–3527. doi:10.1128/AEM.71.7.3524-3527.2005

Richards GP, Watson MA, Crane EJ III, Burt IG, Bushek D (2008) *Shewanella* and *Photobacterium* in oysters and seawater from the Delaware Bay. Appl Environ Microbiol 74:3323–3327. doi:10.1128/AEM.00060-08

Simidu U, Tsukamoto K (1985) Habitat segregation and biological activities of marine members of the family Vibrionaceae. Appl Environ Microbiol 50:780–790

U.S. Environmental Protection Agency (1989) Ambient water quality criteria for ammonia (saltwater)—1989. Publication no. EPA 440/5-88-004. Office of Water Regulations and Standards. U.S. EPA, Washington, DC

Wazniak C, Sturgis B, Hall M, Romano W (2005a) Dissolved oxygen status and trends in the Maryland coastal bays. In: Maryland's coastal bays: ecosystem health assessment. Maryland Department of Natural Resources, Annapolis, pp 47–52

Wazniak C, Sturgis B, Hall M, Romano W (2005b) Nutrients status and trends in the Maryland coastal bays. In: Maryland's coastal bays: ecosystem health assessment. Maryland Department of Natural Resources, Annapolis, pp 5–17

Wazniak C, Wells D, Hall M (2005c) The Maryland coastal bays ecosystem. Chapter 1, Maryland's coastal bays: ecosystem health assessment. Maryland Department of Natural Resources, Annapolis, pp 1.9–1.20

Wright AC, Hill RT, Johnson JA, Roghman MC, Colwell RA, Morris JG (1996) Distribution of *Vibrio vulnificus* in the Chesapeake Bay. Appl Environ Microbiol 62:717–724

Zervanos SM (1978) Summer determination of activity patterns of Assateague ponies by means of long range telemetry. National Park Service, Berlin, MD

Zevanos SM, Keiper RR (1979) Winter activity patterns and carrying capacity of Assateague Island feral ponies. National Park Service, Berlin, MD